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Quantitative PCR analysis of diepoxybutane and epichlorohydrin damage to nuclear versus mitochondrial DNA

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Abstract

The bifunctional alkylating agents diepoxybutane (DEB) and epichlorohydrin (ECH) are linked to the elevated incidence of certain cancers among workers in the synthetic polymer industry. Both compounds form interstrand cross-links within duplex DNA, an activity suggested to contribute to their cytotoxicity. To assess the DNA targeting of these compounds *in vivo*, we assayed for damage within chicken erythro-progenitor cells at three different sites: one within mitochondrial DNA, one within expressed nuclear DNA, and one within unexpressed nuclear DNA. We determined the degree of damage at each site via a quantitative polymerase chain reaction, which compares amplification of control, untreated DNA to that from cells exposed to the agent in question. We found that ECH and the related compound epibromohydrin preferentially target nuclear DNA relative to mitochondrial DNA, whereas DEB reacts similarly with the two genomes. Decreased reactivity of the mitochondrial genome could contribute to the reduced apoptotic potential of ECH relative to DEB. Additionally, formation of lesions by all agents occurred at comparable levels for unexpressed and expressed nuclear loci, suggesting that alkylation is unaffected by the degree of chromatin condensation.

Keywords

Diepoxybutane; Quantitative PCR; DNA damage; Cytotoxicity; Alkylating Agent; Apoptosis

1. Introduction

Bifunctional alkylating agents have played a central role in cancer chemotherapy since the introduction of nitrogen mustards into clinical settings over 50 years ago [1]. The profound cytotoxicity of these agents has been attributed to formation of covalent DNA interstrand cross-links that disrupt normal replication and transcription [2]. While DNA cross-linkers remain

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Supporting information available Complete sequences of PCR products for each locus probed are available.

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widely used as anticancer agents, their use is associated with subsequent development of hematopathologies such as myelodysplastic syndrome and acute myeloid leukemia [3,4,5]. Occupational exposure to cross-linkers, or agents that are metabolized to cross-linkers, can also increase cancer risk. For example, workplace exposure to 1,3-butadiene (BD) has been linked to increased incidence of hematopoietic and lymphatic cancers [6,7,8,9], and exposure to epichlorohydrin (ECH) appears to increase risk of lung cancer [10,11]. We are investigating the mechanisms by which these high-volume industrial chemicals exert their biological effects. While the molecular determinants of the ultimate effect of a cross-linking agent remain to be elucidated, genomic targets are likely to play a role in carcinogenicity versus antitumor potential.

The metabolites of BD include monoepoxides and a diepoxide, diepoxybutane (DEB). DEB is 100-fold more mutagenic and cytotoxic than the monoepoxide metabolites, suggesting that the biological effects of BD exposure may arise principally from the formation of DNA interstrand cross-links [12,13]. DEB reacts with synthetic DNA oligomers to form interstrand cross-links preferentially at 5'-GNC sites [14]. Although the 5'-GNC consensus sequence of DEB is preserved in defined-sequence nucleosomal core particles [15], such model systems may not be completely representative of the sites targeted in the genome. High-level chromatin structure modulates the DNA binding of many mutagens, carcinogens, and anticancer drugs *in vivo* [16]. Indeed, chromatin structure has been proposed to explain the different DEB products observed with free and cellular DNA [17]. Furthermore, the relative contributions of mitochondrial DNA (mtDNA) damage versus nuclear DNA (nDNA) damage to the effects of DEB and other cross-linkers are unknown. Mutations in mtDNA are linked to human diseases such as cancer, the normal process of aging, and the triggering of apoptosis [18,19,20].

Our goal in this study was to assess DNA damage *in vivo* by DEB, the industrial cross-linker ECH, and the related compound epibromohydrin (EBH). Like DEB, ECH and EBH form cross-links between deoxyguanosine residues, although they are less stringent in their sequence requirements, cross-linking 5'-GC and 5'-GNC sites about equally [21]. We used the technique of Quantitative Polymerase Chain Reaction [QPCR], in which the presence of DNA lesions can reduce the amount of PCR product relative to an unmodified template by inhibiting *Taq* polymerase [22]. The lesion frequency at the locus of amplification can be determined through a Poisson analysis of the amplification of the damaged template versus the undamaged template [23]. QPCR has been used to assess DNA damage induced by a wide variety of genotoxins [24]. DEB and the epihalohydrins principally alkylate at the N7 position of deoxyguanosine residues [14,21], leading to heat-sensitive adducts that are likely to undergo cleavage under thermal cycling conditions [25]. QPCR was therefore used to detect total damage, including both monoadducts and cross-links, in this study.

We used QPCR to monitor reactivity of DEB, ECH, and EBH within 6C2 (erythro-progenitor) chicken cells at loci differing in their degree of chromatin condensation. Each locus was approximately 400 base pairs (bp) in length. One locus was within mtDNA (a portion of the cytochrome *b* gene), one was within open, expressed nDNA (a portion of the folate receptor [FR] gene), and one was within highly condensed, unexpressed nDNA (near the β -globin locus). MtDNA lacks any associated proteins, whereas nDNA interacts with histones to form chromatin [26]. Chemical modification of histones, a control mechanism for gene expression [27], modulates histone-DNA interactions and chromatin structure [28] and thereby has the potential to affect reactivity with external agents.

We found that the lesion frequencies for DEB were comparable for all loci, whereas the lesion frequencies for the epihalohydrins were three to four times higher for the nuclear loci than for the mitochondrial one. These findings suggest that DEB partitions about equally between the two genomes *in vivo* but that ECH and EBH target nuclear DNA preferentially. Decreased

targeting of the mitochondrial genome by ECH could contribute to its reduced apoptotic potential relative to DEB. Furthermore, chromatin condensation appeared to have no significant influence on nDNA alkylation by the agents examined.

2. Materials and Methods

2.1 Cell Culture

6C2 (erythro-progenitor) chicken cells were grown in Richter's modified Eagle's medium supplemented with 10% fetal bovine serum, 2% chicken serum, 1 mM HEPES, 50 μ M β -mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C in 5% CO₂.

2.2 Cytotoxicity Assays

The cytotoxicities of DEB and ECH (both as racemic mixtures from Sigma-Aldrich) were determined in 6C2 cells via propidium iodide exclusion [29,30]. (Caution: DEB, ECH, and EBH are suspect carcinogens and must be handled appropriately.) Briefly, confluent cells were plated at a 1:5 dilution with MEM Richter's modification with L-glutamine and grown for at least 18 h (37°C, 5% CO₂) to ensure entry into the log phase of growth. Cells were treated with 0 mM, 2.5 mM, 10 mM, 25 mM, 100 mM, or 250 mM DEB or ECH, and 1-mL aliquots were removed after 15, 30, and 60 minutes. Treated cells were centrifuged at 250 $\times$ g for 5 minutes, and the pellets were washed with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl [pH 7.4]), centrifuged again, and suspended in 1 mL PBS. Ten microliters propidium iodide (from a 50 ng/mL stock in PBS) were added, and samples were covered with aluminum foil and incubated at room temperature for 10-15 minutes. Cell viability was determined via flow cytometry (BD Biosciences FACScalibur with CellQuest software), with propidium iodide emission indicating cell death [29]. The viable fraction was plotted versus concentration, and the Solver tool in Excel was used to optimize the values of a and b in the equation $y=1/[1+(x/a)^b]$ to achieve the best-fit dose response curve, with a being the LD₅₀ (the dose lethal to 50% of the cells). Data were obtained in duplicate or triplicate, and SolverAid [31] was used to determine the standard error for the LD₅₀ value at each time point.

2.3 Treatment with Cross-Linkers

Cells at 85-95% confluence were passaged into a total volume of 5 mL of fresh medium 24 h prior to treatment with cross-linker. To establish appropriate reaction conditions, $1-5 \times 10^6$ cells were incubated with varying concentrations of DEB (2.5 mM — 250 mM) for varying times (15 min — 180 min) at 37°C in 5% CO₂. After initial experiments with DEB, a concentration of 250 mM was used in subsequent QPCR experiments for all cross-linkers. ECH and EBH incubation times ranged from 15 min to 240 min (previous work suggested that longer incubation times would be required for these agents than for DEB [21]). After incubation, cells were transferred to 15 mL conical tubes and the cell culture plates were washed with 5 mL of PBS, which was subsequently added to the tubes containing the cells. Cells were centrifuged at 500 $\times$ g for 5 min, and the resulting cell pellets were washed with 10 mL of PBS. Cells were pelleted again by centrifugation as above and stored at -20°C until DNA isolation.

2.4 DNA Isolation

Cell pellets were resuspended in 300 μ L digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K) and incubated at 50°C for ~15 h. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol followed by ethanol precipitation with 2.5 M ammonium acetate and 10 μ g of glycogen. DNA pellets were washed with 70% ethanol, dried, and resuspended in 100 μ L water. DNA was subsequently ethanol precipitated with 0.3 M sodium acetate, washed with 70% ethanol, dried,

and resuspended in 100 μ L water. DNA was quantitated using a NanoDrop® ND-1000 UV-Vis spectrophotometer.

2.5 PCR Primer Design

All primer pairs amplified circa 400 bp products. Mitochondrial PCR primers were derived from the universal primers H15149 and L14841 [32] but were designed using the program OLIGO (Molecular Biology Insights) to be exactly complementary to the chicken genome. The sequences of these primers were as follows: 5'-CTCCCAGCCCCATCCAACATCTCTGCTTGATGAAA and 5'-TAACGGTGGCCCCCTCAGAATGATATTTGGCCCCA. Primers for the expressed and unexpressed loci were designed based on genomic data and published maps of the region in the vicinity of the β -globin domain [33,34]. For the expressed region, we used a portion of the folate receptor gene, which is located upstream of the β -globin locus and is expressed in the 6C2 cell line [34]. The primer sequences for this locus were 5'-AAAGTACTACGCCTGGAAGAAGAGA and 5'-ATTCAGAAATGGATCATGAACAAAC. For the unexpressed locus, we probed a region of highly condensed chromatin at the 3' terminus of the β -globin gene [33]; primer sequences were 5'-AGTACTGCCGTGTGTTTGCTC and 5'-TACAGCCCTCTCAGCAAGTAA. Amplification of the correct regions was confirmed through sequencing of the products (Supporting Information) and comparison with the *Gallus gallus* genomic data via BLAST [35].

2.6 Quantitative PCR

Initial experiments were performed to determine the linear range of amplification for each locus by varying the amount of template DNA in each PCR reaction. Subsequent PCR reactions (25 μ L) contained 1.25 units of Taq DNA polymerase, 1X Taq buffer B (Fisher Bioreagents), 2.5 mM $MgCl_2$ (3.0 mM for the FR locus), 0.2 mM each dNTP, 0.5 μ M each primer, 1 μ Ci [α - ^{32}P]dATP, and 2 ng template DNA. Nuclear DNA was amplified via a “touchdown” protocol [36] that consisted of the following thermal cycling sequence: 5 min at 95°C; 10 cycles of 30 s at 95°C, 30 s at 65°C (decreased by 1°C each cycle), and 30 s at 72°C; 20 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; 10 min at 72°C. Mitochondrial DNA was amplified via a “two-step” protocol: 5 min at 95°C; 25 cycles of 30 s at 95°C and 30 s at 70°C; 10 min at 72°C. Following thermal cycling, 5 μ L loading dye (0.25% xylene cyanole in 40% sucrose) was added to each PCR reaction. A 10- μ L aliquot of each PCR reaction was analyzed via 8% native polyacrylamide gel electrophoresis (PAGE) at 200 V for circa 1.5 h at 4°C after pre-running for 1.5 h. Gels were subsequently dried, exposed to phosphorimager screens, and quantitated using a Storm 840 phosphorimager (Molecular Dynamics).

2.7 Poisson Analysis of QPCR Products

Lesion frequencies at various loci were calculated through a Poisson expression analysis. Lesions per strand were calculated as the negative natural logarithm of the ratio of the intensity of the QPCR product from the damaged DNA to the intensity of the QPCR product from undamaged DNA [23]. This value was then converted to lesions per kbp by correcting for the length of each PCR product (unexpressed locus, 398 bp; expressed locus, 451 bp; mitochondrial locus, 376 bp). Values for lesions per kbp were plotted versus treatment time, with lesion formation frequency calculated from the slope of the best-fit line for the linear part of the curve [24]. Data from at least three trials were averaged.

2.8 Assessment of Repair of ECH Lesions

Cells at 85-95% confluence were passaged into a total volume of 5 mL of fresh media and incubated at 37°C and 5% CO_2 for at least 18 h to ensure entry into the log phase of growth.

Samples were then treated with 250 mM of ECH for 4 h at 37°C and 5% CO₂. Following incubation, samples were centrifuged at 250 × g for 5 min at room temperature. The supernatant was removed and the pellet was re-suspended in 5 mL cold 1X PBS. The centrifugation was repeated and the pellet was re-suspended in 5 mL fresh medium. Cells were re-plated and incubated at 37°C and 5% CO₂ for time intervals of 0 h-24 h. Following this incubation, DNA was isolated and QPCR was performed as described above.

2.9 Assessment of Apoptosis via Caspase Activity Assays

Caspase-3/7 activity was monitored in cells treated with 250 mM DEB or ECH for various times to assay for apoptosis. Briefly, confluent 6C2 cells were plated at a 1:5 dilution with MEM Richter's modification with L-glutamine and grown for at least 18 h (37°C, 5% CO₂). Cells were treated with DEB (250 mM or 50 μM), ECH (250 mM or 50 μM), or 1 mM camptothecin (positive control for apoptosis [37]) and 100 μL aliquots were removed after various incubation times. Samples were centrifuged at 250 × g for 5 minutes, and the pellets were washed with PBS, centrifuged again, and suspended in 100 μL PBS. Samples were transferred to a 96-well plate, and 100 μL of caspase-3/7 reagent (Promega) was added. Caspase activity was measured by luminescence continuously (Molecular Devices Spectra Max M2) for circa 90 min.

3. Results

3.1 Determination of Cross-Linker Cytotoxicity in 6C2 Chicken Cells

In order to determine appropriate doses of cross-linkers, the cytotoxicities of DEB and ECH were determined in 6C2 chicken cells. These rapidly growing cells have a doubling time of about 8 h. Therefore, we chose to use relatively high cross-linker doses and short incubation periods to minimize the effects of DNA replication and repair, which would conceal cross-linker-induced damage, as well as apoptosis, which would enhance damage. LD₅₀ values, determined via propidium iodide staining and flow cytometry (Figure 1, Table 1), were comparable to concentrations commonly used for optimal DEB- and ECH-induced DNA cross-linking [14,38,21]. Although bifunctional alkylating agents react with a number of biological nucleophiles, DNA cross-linking is generally considered to be the most lethal event [2]. DEB and ECH are inefficient cross-linkers, requiring doses three orders of magnitude higher than those needed for nitrogen mustard to achieve comparable levels of cross-linking [39,21].

3.2 QPCR Analysis following DEB Treatment

To assess DEB reactivity in chicken erythro-progenitor cells, QPCR with different primer pairs was used to monitor three distinct regions of DNA, each about 400 bp in size. Micrococcal nuclease digestion and Southern blotting [40] were used to confirm literature reports of the degree of condensation at the nuclear loci (FR locus: uncondensed [34]; β-globin locus: condensed [33]; data not shown). Preliminary PCR experiments were performed with differing template concentrations and numbers of cycles to determine the linear amplification range for each product. Cultured cells were then treated with DEB, and total DNA was isolated and used as the template for QPCR. Equal amounts of DNA from untreated (control) and DEB-treated cells were amplified independently in the presence of radiolabeled dATP. Incorporation of radiolabel into full-length product was dependent on the integrity of the template and therefore reflected the degree of damage under each set of DEB reaction conditions.

In general, analysis of QPCR reactions by 8% native PAGE revealed decreasing amounts of all PCR products with longer DEB incubation times (Figure 2). The product bands were quantified to determine the relative amount of amplification (the ratio of amplification of damaged template to amplification of undamaged [control] template). We then calculated the lesion frequency at each locus through Poisson expression analysis, used to describe the

frequency at which a random event is occurring to a population [23]. According to the Poisson equation, lesions per DNA strand = $-\ln(A_D/A_C)$, where A_D/A_C is the relative amplification (ratio of damaged to control amplification).

Varying the concentration of DEB (2.5 mM, 25 mM, and 250 mM) at a constant incubation time of 1 h revealed reproducibly detectable lesions only at the highest concentration (Figure 3). Furthermore, the average lesion formation frequency increased linearly with increasing concentration. We therefore used 250 mM DEB for subsequent experiments. Although this dose is quite high, DEB and ECH are both highly inefficient cross-linkers compared to nitrogen mustard, mitomycin C, and other common bifunctional alkylating agents [14,21].

Analysis of time-course data revealed a relative amplification of less than 10% for all PCR products after 120 minutes of treatment with 250 mM DEB (Figure 4A). An exposure time of 180 minutes virtually completely inhibited amplification of PCR products. Lesions increased linearly over time for all three loci up to 180 minutes (Figure 4B), suggesting that damage events were random and independent during this time period. Lesion formation frequencies of circa 3 lesions/h/kbp were comparable to previous reports of mustard damage in HeLa cells [41]. Furthermore, there was no significant difference in lesion formation frequency between the different loci (Table 2), suggesting comparable damage to nuclear and mitochondrial DNA by DEB.

3.3 QPCR Analysis following Epihalohydrin Treatment

To assess ECH and EBH reactivity, we used QPCR with the same primer pairs. Cultured cells were treated with 250 mM ECH or EBH for varying lengths of time, and total DNA was isolated and used as the template for QPCR. In contrast to DEB, the epihalohydrins displayed a decreased lesion formation frequency at the mitochondrial locus relative to the nuclear loci (Table 3). However, the lesion frequencies for the two different nuclear loci were similar.

3.4 Assessment of Mitochondrial Repair of ECH Lesions

QPCR was also used to monitor repair of damage to mtDNA by ECH. 6C2 cells were treated with 250 mM ECH for 4 h, a time period earlier found to induce significant damage (relative amplification of circa 10%). Following removal of ECH, cells were allowed to recover for various repair time intervals (up to 24 h). DNA was isolated and subjected to QPCR. Data were analyzed via the Poisson expression and corrected for length of PCR product in order to determine lesions/kbp as a function of repair time (Figure 5). The mitochondrial locus appeared to undergo periodic repair, with lesions/kbp decreasing and then increasing again in cyclical manner. In contrast, the number of lesions within the nucleus remained constant over the same time period, revealing no evidence of repair.

3.5 Assessment of Apoptosis via Caspase Activity Assays

In order to verify that apoptotic events were not contributing to the observed DNA damage, we monitored for caspase-3/7 activity within cells treated with DEB or ECH under the conditions used for QPCR. This assay involves a proluminescent caspase-3/7 substrate, which generates a luminescent signal in the presence of caspase-3 or -7. Although the positive control (1 mM camptothecin, 4 h treatment) displayed high luminescence, 6C2 cells treated with 250 mM DEB or ECH for up to 4 h did not (Figure 6A). Cells treated with 50 μ M DEB did show luminescence after 21 h, but those treated with 50 μ M ECH did not (Figure 6B).

4. Discussion

Previously, we reported the consensus sequences for DEB and epihalohydrin cross-linking within DNA oligomers [14,21]. Both agents cross-link deoxyguanosines on opposite strands

at 5'-GNC sites, where N is any base. Unlike DEB, the epihalohydrins cross-link 5'-GC as effectively as 5'-GNC sites. While the 5'-GNC cross-linking preference of DEB is conserved in defined sequence core particles [15], bases flanking the core sequence modulate cross-linking efficiency [39], suggesting that specific genomic regions could be targeted with some degree of selectivity. Our goal was to discern information about the genomic sites damaged by DEB and the epihalohydrins in order to provide insight into the molecular mechanisms of carcinogenesis. In addition to sequence effects, access to the genetic material, as modulated by compartmentalization and chromatin structure, could influence reactivity. We used QPCR to determine the amount of damage in mtDNA and two different nuclear loci. Because the use of chemotherapeutic alkylating agents is associated with subsequent development of hematopathologies such as leukemia [3,4,5], we used erythro-progenitor (chicken 6C2) cells for our studies.

In eukaryotic cells, the majority of nDNA exists as chromatin, a polymer composed of repeating "nucleosome" subunits (for reviews, see [26] and [42]). Nucleosomes are composed of a core particle, closely associated with the histones, and a linker region. The binding of many external agents to nucleosomal DNA is modulated by rotational and translational positioning along the core particle, and linker DNA often has greater reactivity than core DNA [16]. Heterogeneity in nitrogen mustard cross-linking within different genes of the same cell line and within the same gene in different human tumor cell lines has also been attributed to variations in chromatin structure, with highly expressed genes undergoing more damage because of increased DNA accessibility [43].

In contrast to nDNA, the human mitochondrial genome is a double-stranded, circular DNA of 16,569 bp with no associated proteins to protect it from damage [44]. Because most of the mitochondrial genome consists of coding sequences, alkylating agents are more likely to damage vital coding regions in the mitochondria than in the nucleus.

Recently, there has been interest in determining the relative importance of DNA damage to the nuclear versus the mitochondrial genome. Although some mutagens, including the most potent metabolite of benzo[a]pyrene [45], cisplatin [46], and aflatoxin [47], bind preferentially to mitochondrial DNA, the cytotoxicity of others, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), appears to involve nDNA and mtDNA about equally [48]. Damage to mtDNA, such as that induced by the insult of xenobiotics, has been linked to apoptosis [49]. Our studies showed that DEB induces comparable damage to nDNA and mtDNA, consistent with a role of apoptosis in its mode of action. Indeed, DEB has been demonstrated to induce caspase-mediated apoptosis at lower concentrations (10 – 75 μ M) and longer incubation times (24 h) than those used in our QPCR studies [50,51,52]. Our caspase activity assays suggested apoptosis in 6C2 cells treated with low-dose DEB (50 μ M) for long incubation times but not in cells treated with high-dose DEB (250 mM) for short times (Figure 6). These observations are consistent with literature reports of an apoptotic window, in which relatively high doses of DEB induce necrosis rather than apoptosis [52], and suggest that the DNA damage that we did observe in the QPCR studies was a direct result of chemical treatment rather than self-destruction by the cell.

Unlike DEB, the epihalohydrins showed a three- to four-fold preference for nDNA relative to mtDNA. Interestingly, ECH has a lower apoptotic potency than DEB [52], an observation that could be related to its reduced targeting of the mitochondrial genome. Indeed, necrosis, rather than apoptosis, has been proposed to be the primary mechanism of death for ECH-treated cells [53]. Our caspase assays support this hypothesis, with no evidence of apoptosis in 6C2 cells treated with ECH under any of our incubation conditions (Figure 6).

Possible explanations for the differential alkylation by ECH and EBH of the nuclear versus the mitochondrial genome include differences in uptake, number of target sites, or repair. Bulky, lipophilic DNA damaging agents have been shown to target mitochondrial DNA because of the high lipid-to-DNA ratio of the mitochondria [45]. This would suggest that poor mitochondrial uptake of the small, relatively polar epihalohydrins¹ could account for their decreased lesion formation frequency. In contrast, the nuclear pore complexes allow free passage of small molecules into the nucleus [54,55]. Furthermore, the nuclear envelope breaks down with every turn of the cell cycle, rendering the nuclear lumen continuous with the cytosol. In contrast, the mitochondrial membrane remains intact throughout the cell cycle, limiting the mitochondrial genome's exposure to xenobiotics in the cytosol.

Both DEB and the epihalohydrins target deoxyguanosine residues preferentially [56,57,58, 59]. Therefore, base composition could impact the relative amount of damage sustained by nuclear and mitochondrial DNA if one genome has fewer target sites. For example, sulfur mustard produces more total adducts in human nDNA than mtDNA, attributed to the nuclear genome's enrichment in runs of consecutive deoxyguanosines, which are preferred by mustards [60]. Furthermore, the lower GC content of mtDNA has been suggested to account for DEB's preferential damage to nDNA in yeast cells [61]. However, our loci do not significantly differ in their GC content (Table 4), although GC content does not entirely reflect abundance of target sites because of minor reactions with adenine *in vivo* by these agents [13,17,62,63]. Nonetheless, DEB cross-linking is more specific than that of the epihalohydrins [21], making it unlikely that the latter compounds would find fewer targets in the mitochondrial genome than in the nucleus given that DEB lesions are comparable for the two genomes.

Finally, more efficient repair of ECH lesions in the mitochondria could also explain the lower lesion frequency. However, the similar chemistry of ECH and DEB makes it unlikely that repair processes would differ dramatically for the two lesions. Although we observed recovery of mtDNA after ECH treatment in terms of fewer lesions over time, this could arise from replication of the mitochondrial genome rather than repair [64]. The mitochondrial genome has a high turnover rate to limit oxidative damage caused by the byproducts of oxidative respiration [49]. Furthermore, lesion frequency first increased in the mitochondria before it decreased. The observed cyclic increase in damage could arise from continued slow uptake of ECH by the mitochondria despite its removal from the extracellular environment. Our QPCR experiments could also be detecting increased oxidative damage to mtDNA because of increased energy needs of the damaged cells.

With respect to the nuclear loci, our results are further support that the degree of DNA condensation does not affect its accessibility to cross-linkers. These findings are consistent with our previous results for the reaction of DEB with nucleosomal core particles [15], supporting a dynamic model of chromatin that allows DNA alkylation by many agents despite packaging with histones [16,65,66].

In conclusion, we have found that DEB targets nDNA and mtDNA equally, but the epihalohydrins have a three- to four- fold preference for nDNA, even at relatively high concentrations. Studies using quantitative real-time PCR to monitor damage at lower concentrations of these agents are currently underway in our laboratory. Further work is also necessary to assess the consequences of damage to each genome to the carcinogenic process, including the role of apoptosis versus necrosis in cell death under varying conditions of concentration and exposure time and the partitioning of cross-links, rather than total damage, within the cell.

¹Dipole is 3.76 for ECH and 3.79 for EBH versus 1.76 for DEB, as calculated via the Hartree-Fock method with a 6-31G* basis set in Spartan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative survival curve for 6C2 cells treated with DEB (15 min incubation). Viable cells were determined by a propidium iodide exclusion assay as described in Materials and Methods. LD₅₀ values were calculated from the best-fit line (Table 1).

Figure 2.

Representative native PAGE analysis of QPCR to monitor reaction of DEB (250 mM) in 6C2 cells over time. A) nDNA (unexpressed region 3' to the β -globin gene); B) mtDNA.

Figure 3.

Representative plot of concentration dependence of DEB-induced DNA damage as assessed by QPCR. Cells were incubated with DEB at concentrations of 2.5 mM, 25 mM, or 250 mM for 1 h, after which time the DNA was purified and subjected to QPCR at the unexpressed locus (region 3' to the β -globin gene). Lesion per kbp were calculated via the Poisson expression, correcting for the size of each PCR product. Data are the averages of six trials per concentration with standard deviations reported as error bars.

Figure 4.

QPCR results after DEB treatment (250 mM) with varying time (unexpressed nDNA [-■-]; expressed nDNA [-●-]; mtDNA [-◇-]). Values are the averages of multiple trials with standard deviations reported as error bars. A) Relative amplification versus time; B) Poisson analysis of DNA damage. Lesion formation frequencies for each locus were calculated from the slope of the best-fit line for the linear region of these plots.

Figure 5. ECH lesion frequency following time for repair. Values are the averages of four trials with standard deviations reported as error bars. A) Mitochondrial locus; B) Unexpressed locus; C) Expressed locus (note the different time scale).

Figure 6.

Analysis of apoptosis in 6C2 cells via luminescence (relative light units, RLU) over time.

Panel A) Cells treated with 1 mM camptothecin (4 h [-○-]), 250 mM DEB (1 h [-□-], 2 h [-△-], and 4 h [-◇-]), or 250 mM ECH (1 h [-■-], 2 h [-▲-], and 4 h [-◆-]). **Panel B)** Cells treated with 50 μM DEB (4 h [-◇-], 21 h [-○-]) or 50 μM ECH (4 h [-◆-], 21 h [-●-]). Luminescent signal is proportional to caspase-3/7 activity.

Table 1

Average LD₅₀ concentrations with standard deviations for DEB and ECH in 6C2 cells as determined through a propidium iodide exclusion assay.

Treatment time (min)	DEB LD ₅₀ (mM)	ECH LD ₅₀ (mM)
15	190 ± 18	182 ± 13
30	91 ± 10	121 ± 8.0
60	57 ± 3.6	48 ± 2.4

Table 2

Average DEB lesion formation frequencies with standard deviations for all loci.

Locus	3' to β -Globin (unexpressed)	Folate Receptor (expressed)	Mitochondrial
Lesion Formation Frequency (lesions/h/kbp)	3.8 ± 0.8	3.1 ± 0.1	3.5 ± 0.5

Table 3

Average epihalohydrin lesion formation frequencies with standard deviations for all loci.

Locus	3' to β -Globin (unexpressed)	Folate Receptor (expressed)	Mitochondrial
ECH Lesion Formation Frequency (lesions/h/kbp)	5.5 ± 0.5	4.9 ± 0.9	1.4 ± 0.1
EBH Lesion Formation Frequency (lesions/h/kbp)	3.4 ± 0.7	3.7 ± 0.1	1.3 ± 0.1

Table 4

GC content of each locus.

Locus	3' to β -Globin (unexpressed)	Folate Receptor (expressed)	Mitochondrial
GC content	43%	57%	53%